MOLECULAR ECOLOGY OF *CRYPTOCOCCUS NEOFORMANS* IN THE MALTESE ISLANDS

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INTRODUCTION

Cryptococcosis is the most life-threatening deep seated fungal infection in AIDS patients. In immunocompetent hosts cryptococcosis is rare. However its effects are devastating, in both immunocompetent and immuno-suppressed patients resulting in death or permanent brain damage, unless cases are diagnosed and treated at an early stage. In Aids a growing problem in the management of secondary diseases is the recurrence of cryptococcosis due to the reactivation of latent strain of C. neoformans probably from prostatic foci.

Many questions related to the ecology and epidemiology of *Cryptococcus neoformans*, the aetiological agents of cryptococcosis, are still unanswered. This extremely versatile fungus is represented by two varieties and five serotypes with different geographical distributions and different epidemiological significance.

C. neoformans variety *neoformans* serotype A has a world wide distribution and it is prevalent in AIDS patients. The serotype D of the same variety has been isolated in Italy, Denmark and France from clinical samples of AIDS patients. (Kwon-Chung, Bennett 1984; Dromer, Varma et al. 1994). Extreme genetic variability among both serotype A and D has been demonstrated by molecular typing techniques. (Meyer, Perfect 1993, Varma 1992).

The variety *gatti* has been isolated in Africa where it is prevalent, in western Australia and in California, leading to the theory that the distribution of *Cryptococcus* varieties are related to regional climatic conditions. The isolation of both environmental and clinical strains is correlated for variety *gattii* with the presence of *Eucalyptus* tree which is the reservoir for this fungus variety. (Ellis and Pfeiffer 1990).

Beside a different geographical distribution the two varieties possess a different degree of pathogenicity and a different sensitivity to antifungal therapy. In fact the variety *gatti* is predominatly a pathogen, the variety *neoformans* is considered opportunistic. However the opportunistic nature of neoformans variety is still being queried.

Information about epidemiology and ecology of *C. neoformans* is lacking in the Mediterranean area. We chose the Maltese Islands for their secluded and strategic position in the centre of the Mediterranean. Malta being, at the

crossroads between Europe and Africa, could be a linking point between the two different ecological patterns of *C. neoformans.* The isolated position of the two islands represents an ideal restricted geographical area for the study of genetic variability between strains.

The aim of these studies is to elucidate the incidence of *C. neoformans* in the Maltese Islands, the prevalence of serotypes and the distribution of genetic relatedness within strains in a limited geographic area.

MATERIALS AND METHODS

Isolates

Environmental strains were isolated from different locations in the Maltese Islands (fig. 1) with the aid of Brown Colour Effect-BCE on Staib Agar supplemented with penicillin G (20 U/ml), streptomycin sulphate (40 U/ml) biphenyl 0.01 and 0.1% (tab.1)

Clinical strains were isolated using SDA + 50 ug/ml chloramphenicol and gentamicin (50 ug/ml). All isolates were identified according to established morphological, cultural and biochemical procedures (Kreeger van Rij, 1984, ATB 32C, Biomerieux, Marcy l'Etoile, France). Variety of C. neoformans isolates was studied on CGB-Agar (8).

Serotyping

Environmental and clinical isolates were grown on Sabouraud Dextrose Agar-SDA (Difco Lab., Detroit, Mich.) at 26C for 48 h. Serotyping (slide agglutination test) was carried out by monoclonal antibodies specific for capsular polysaccharide (Crypto chech-Iatron Lab.Japan).

Molecular approach

Environmental and clinical isolates

Twelve out of twenty environmental strains (selected according to site of isolations and two clinical isolates were used in this study (tab 2).

Growth conditions

Strains were grown in potato dextrose broth (Difco, Detroit, Mich.) at 37C with shaking for 48 h.

DNA isolation

Isolation of genomic DNA was based on the method of Restrepo and Barbour (19). Two hundred-milliliter cultures in stationery phase (OD 600 = 1.3) were harvested by centrifugation (5,000g for 5 min at 4 C). The cells were suspended in 20 ml of sol A, 10 mM Tris (pH7.5), 50 mM EDTA, 10 mM dithiothreitol and incubated for 15 min at 30C while shaking gently. Cells were centrifuged (5,000g for 5 min) and washed with 20ml of CPE buffer 40mM citric acid, 120 mM Na2HPO4, 50mM EDTA(pH=6). The pellet was resuspended in 3ml of sol. B (CPE buffer with 1,2M sorbitol and 5mM dithiothreitol) and warmed for 5 min at 38 degree C. The enzymatic lysis was achieved by adding 20 mg of "Lysing enzymes" from Trichoderma harzianum

(L2265-Sigma) in 5 ml of CPE buffer. The reaction mixture was incubated overnight at 30 degree C shaking gently. Formation of spheroplasts was confirmed by microscopy. The cells were then spun down (5,000xg for 5 min) and the pellet was suspended in 8,5ml of NDS [50ml EDTA, 10mM Tris (pH 7,5), 1% (vol/vol) sodium dodecylsulfate] with 0,5mg/ml of proteinase K. The suspension was incubated first for 1h at 37 degree C and then for 15 min at 65 degree C. Unbroken cells and cell wall debris were removed by centrifugation at 6,000xg for 5 min. The supernatant was cooled on ice for 10 min and then 1ml of 5M potassium acetate was added .The suspension was placed on ice for 20 min and centrifuged at 16,000xg for 10 min at 4 degree C. 2ul of RNase solution (10mg/ml) was added and the reaction volume was incubated for 30 min at 37 degreeC .The suspension was extracted with a volume of phenol chloroform(1:1)solution (pH 8) and then with a volume of chloroform . Nucleic acid was precipitated with 2,5 volumes of cold ethanol for 1 hour at -80C. The precipitate was recovered by centrifugation for 30 min at 10,000 g at 4C and then suspended in 1ml of TE (10 mM Tris ph 8, 1mM EDTA).

PCR Enzymatic amplification of DNA

PCR reactions were performed on an automated thermocycler Biostar MR4 (violet) in a 100 ul reaction volume containing 10 ng of template DNA, 0,2 mM of each deoxynucleoside triphosphate (dNTP) (Pharmacia LKB Biotechnology), 20 ng of primer, 3 mM CH3COOMg, added only for the method of Meyer et al., 1993 (13) and 0,5U of super TAQ DNA polymerase (HT Biotechnology LTP, England) using buffer conditions recommended by the manufacturer.

RAPD were performed by methods of Meyer et al., 1993 (13) and Crampin et al., 1993 (3). The microsatellite DNA sequence (GTG)5, (GACA)4 and the phage M13 core sequence (GAAGGGTGGXGGXTCT) were used as oligonucleotide primers to perform the method of Meyer. PCR was carried out for 40 thermal cycles, each consisting of 20s of denaturation at 93 degree C ,60s of annealing at 50 degree C and 20s of primer extension at 72 degree C, followed by a final extension cycle for 6 min at 72 degree C.

The 8 mer (GCGCACGG) oligonucleotide primer was used in the Crampin method . The reaction mixture was heated to 95 degree C for 10 min. and then the TAQ polymerase was added. Amplification conditions were: 45 thermal cycles, each consisting of 1 min at 96 C, 2 min. at 30 C and 2 min. at 72 C, with a final extension time of 7 min at 72 C . PCR fingerprint products were analysed by electrophoresis in 1,4% agrarose gel run in Tris-Borate-EDTA buffer, and detected by staining with bromine under UV light.

RESULTS

Eighteen out 82 samples of pigeon droppings (21,9%) and 2 out 47 samples of *E. camaldulensis (4,2%)* were positive for C. neoformans (tab.1).

Cfu of positive samples ranged from $4x10^2$ to $>3x10^7$ (tab.2).

Variety: All environmental and clinical isolates of C. *neoformans* tested for variety on CGB agar belonged to var. *neoformans.* According to Kwon Chung et al.,1982(8) only reference strains CDC 3175 ser. B and NIH 18 ser. C produced alkalinization of the CGB agar medium (blu cobalt color).

Serotyping: All environmental and clinical *C. neoformans* var. *neoformans isolated* from Maltese Islands belonged to serotype A . No untypable strains were found .

RAPD fingerprinting

Amplification product sizes obtained ranged from 300 to 1.8 kb. The number of fragments obtained by PCR was dependent on the primer: (GTG)5 generated from 4 to 9 fragments (fig.2), while (GACA)4 produced from 4 to 11 fragments (fig.3), M13 core sequence primer produced 18 fragments (fig.4) and GCGGACGG showed from 7 to 9 fragments (fig .5). Fragments less represented or doubtfully interpreted were not assumed as pattern discriminate elements. Analysis of the PCR patterns allowed us to detect the variability among *C . neoformans* strains at individual level. The employed primers allowed us to amplify and analyze different portions of DNA sequence, resulting in 10 different electophoretic profiles:2 by (GTG)5 (arbitrarly named I and II);2 by (GACA)4 (named III and IV), 2 by M13 (named V and VI); 4 by GCGGACGG (VII,VIII,IX and X) (tab.3).

(GTG)5 primer amplification yielded two different electrophoretic profile classes : the class I - (GTG)5 consists of 13 strains (Cn40M1,Cn47M1, Cn72M3, Cn74M2, Cn74M3, Cn13EM1,Cn50M1, Cn79M1, Cn108M1, Cn111M1, Cn99M1, CnMa, Cn2) while the class II - (GTG)5 was represented by only one strain (Cn41M1) with only 4 fragments.

(GACA)4 primer showed a low amplification efficiency with two electrophoretic profile classes [class III-(GACA)4. and class IV-(GACA)4]. The two electrophoretic profile classes differing each from other by the presence [class III-(GACA)4 : Cn41M1, Cn47M3, Cn13EM1, Cn50M1, C79M1, Cn108M1, Cn99M, CnMa, Cn2] or absence [class IV- (GACA)4: Cn40M1, Cn72M3, Cn74M3, C111M1] of a 1.800 bp fragment.

The M13 core sequence amplification yielded two different electrophoretic profiles (classes V- M13 and VI- M13). These two classes are discriminated by a 450 bp fragment that was present in strains : $Cn40M^1$, $Cn41M^1$, $Cn47M^1$, $Cn13EM^1$, $Cn79M^1$, $Cn111M^1$, $Cn99M^1$,CnMa, Cn2(class V- M13) and absent in strains: $Cn72M^3$, $Cn74M^3$, $Cn50M^1$, $Cn108M^1$,(class VI- M13).

The amplified DNA obtained by GCGGACGG (fig 4) allowed us to distinguish between four electrophoretic profile classes (class VII-Cram, VIII-

Cram, IX-Cram and X-Cram). The class VII-Cram consists of 7 strains (Cn41 M^1, Cn47M^1, Cn13EM^1, Cn111M^1 , Cn99M^1, CnMa, Cn2) showing two fragments of - 450bp; the class VIII-Cram was represented by 3 strains (Cn74M^3, Cn50M^1, Cn108M^1) with a furthur - 500bp fragment; the class IX-Cram consists of 2 strains (Cn72M^3 and Cn74M^2) showing the absence of a - 1.100bp fragment and the class X-Cram represented by 2 strains (Cn40M^1 and Cn79M^1) with a single 450bp fragment.

Including in the same phenotypic class strains having the identical electrophoretic profiles it was possible to differentiate 8 classes (A,B,C,D,E,F,G,H). Class A, for example, included all the strains that showed the pattern I by (GTG)5 primer , the pattern IV by (GACA)4 primer, the pattern V by M13 and the pattern X by GCGGACGG.

DISCUSSION

The majority of C. neoformans isolates in the Maltese islands came from pigeon droppings; only two strains were isolated from E. camaldulensis samples, that could represent a contamination by pigeon droppings.

The high number of C. neoformans in pigeon droppings in the Maltese islands indicate that if the birds could be the direct reservoir of the fungus, some localities in Malta must be regarded as a high level of risk for the inhalation of the fungus and a danger for the immunocompromised and AIDS patients.

The importance of serotype determination and strains variability in C. neoformans is dictated by the high degree of failure in Cryptococosis management in both immuno-suppressed and immunocompetent patients. The increasing number of AIDS patients effected by meningeal cryptococcosis caused by C. neoformans serotype A create the necessity for a serotype determination of Cryptococcus strains.

Malta isolates all belong to the serotype A, including these islands in the European pattern for Cryptococcus ecology and cryptococcosis epidemiology. The geographic and climatological conditions seem in fact to play an important role in serotype distribution.

Sources of clinical isolates included two cases of fatal meninigeal cryptococcosis in two AIDS victims which could explain the prevalence of serotype A in the clinical strains. C. neoformans serotype A is in fact the most frequent serotype isolated from AIDS patients, the other serotypes have been only sporadically isolated.

The frequent recurrences of meningeal cryptococcosis poses the question if it is a reinfection or a reactivation of latent strains. For this reason the relatedness and variability of strains of the same serotype could play an important role. Since serotyping with monoclonal antibodies did not provide enough

variability, it was necessary to employ techniques of molecular fingerprinting which are able to differentiate at strain level.

Strain variation among Cryptococcus neoformans serotype A isolates have been demonstrated by using biochemical, immunological and molecular typing techniques. A molecular approach based on RAPD method was chosen to discriminate at individual level in a population serotypically indistinguishable. The DNA fingerprinting generated with RAPD methods resulted in a wide variability of patterns that has been helpful to distinguish between isolates.

In the Maltese strains the extensive genetic diversity among environmental strains and the close relatedness of the clinical strains were striking findings. Both the clinical strains in fact belong to the "C class" which was also the most common class in the environmental isolates.

The relatedness between clinical isolates and some environmental strains could be regarded as a confirmation that the pigeon droppings are the source of infection. If this hypotesis will be confirmed preventive measures should be suggested to protect Aids patients from acquired cryptococcosis.

These results confirm the presence of quite a wide heterogeneity within environmental strains of *C. neoformans* serotype A in a restricted area. When we tried to fit either the phenotypic classes or a single pattern to the isolation sources we did not find any specific pattern associated with the origin of the environmental strains. These findings are in accord with Currie et al. who detected the same high variability in strains isolated from a limited geographic area in New York City (Currie B.P. et al.1994). The same high variability in strains of serotype D has been detected by Dromer et al.(1994) in a national survey using clinical samples and carried out at the Pasteur Institute, Paris, France.

The relatedness of our clinical samples is very interesting but due to the limited number of strains it is impossible to draw any conclusion about the possibility of finding some molecular pattern for use as a molecular marker in the epidemiology of cryptococcosis. A large number of clinical samples are required to confirm such an intriguing hypothesis.

REFERENCES

1) **Bennet J.E., Kwon-Chung K.J., Howard D.H.** 1977Am.Journ. of Epidemiol.105:582-586.
2) **Casadevall A.,Freundlich L.F., Marsh L., and Scharff M.D.**1992 J.Clin. Microbiol. **30**: 1080-1084.
3) **Crampin A.C, Matthews R.C, Hall D . and Evans E.G.V.**1993 J. Medical and Veterinary Mycology 31:463-465.
4) **Criseo G., Messina S., Chidemi G.** 1992 Atti 1 Congr. Naz. Fed. Ital. Micopatol Umana e Animale (FIMUA) AIMP-SIMUA,Firenze,26-28 Sept.

5) **Criseo G., Bolignano M.S., De Leo F., Staib F. 1995** Zbl. Bakt.: 282: 244-254 (in press)
6) **Currie B.P., Freundlich L.F. and Casadevall A.** 1994 J. Clin Microbiol. 32: 1188-1192
7) **Dromer F.,Varma A., Ronin O.,Mathoulin S. and Dupont B.**1994 J Clin. Microbiol. 32:2364-2371.
8) **Kwon-Chung K.J., Bennett J.E. and Rhodes J.C.**1982 Antonie Van Leeuwenhoek 48:25-38.
9) **Kwon-Chung K.J. and Benbett J.E.**1984. American Journal of Epidemiology. 120:123-130.
10) **Kwon-Chung K.J. and Rhodes J.C.** 1986 Infect. Immun.51:218-223.
11) **Kwon-Chung K.J. and Bennett J.E.**1992 Med. Mycol. 397-446.Edited by Lea & Febiger.
12) **Fan M., Currie B.P., Gutell R.R., Ragan M.A. and Casadevall A.**1994J. Medical and Veterinary Mycology 32:163-180.
13) **Meyer W., Mitchell T.G., Freedman E., Vilgalys R.** 1993 J. Clinical Microbiol. 31:2274-2280.
14) **Perfect J.R., Magee B.B. and Magee P.T.** 1989 Infect. Immun.57:2624-2627.
15) **Perfect J.R., Ketabchi N., Cox G.M., Ingram C.W., and Beiser C.L.**1993 J.Clin.Microbiol..31:3305-3309.
16) **Pfeiffer T.J. and Ellis D.H.** 1992 J.Med.Vet. Mycol.30:407-408.
17) **Pfeiffer T.J. and Ellis D.H.** 1993 J.Med. Vet.Mycol.31:4401-404.
18) **Polacheck I., and Lebens G.I.** 1989 J. Gen.Microbiol.135:65-71.
19) **Restrepo I.B. and Barbour A.G.**1989 J. of Bacteriology 171:596-5600.
20) **Varma A., and Kwon-Chung K.J.**1989 J. Gen. Microbiol. 135:3353-3362.
21) **Varma A. and Kwon Chung K.J.** 1992 J. Clin. Microbiol. 30:2906-2967.
22) **Vilgalys R.and Hester M.** 1990 J.of Bacteriology 172:4238-4246.
23) **Wickes B.L., Moore T.D.E. and Kwon-Chung K.J.** 1994 Microbiology 140:543-550.